A High-Throughput Ligand Competition Binding Assay for the Androgen Receptor and Other Nuclear Receptors

CLÉMENTINE FÉAU, LEGGY A. ARNOLD, AARON KOSINSKI, and R. KIPLIN GUY

Standardized, automated ligand-binding assays facilitate evaluation of endocrine activities of environmental chemicals and identification of antagonists of nuclear receptor ligands. Many current assays rely on fluorescently labeled ligands that are significantly different from the native ligands. The authors describe a radiolabeled ligand competition scintillation proximity assay (SPA) for the androgen receptor (AR) using Ni-coated 384-well FlashPlates® and liganded AR-LBD protein. This highly reproducible, low-cost assay is well suited for automated high-throughput screening. In addition, the authors show that this assay can be adapted to measure ligand affinities for other nuclear receptors (peroxisome proliferation-activated receptor γ, thyroid receptors α and β). (*Journal of Biomolecular Screening* 2009:43-48)

Key words: scintillation proximity assay, androgen receptor, high-throughput screening, endocrine disrupting chemicals, nuclear receptors

INTRODUCTION

The androgen receptor (AR) mediates androgen functions, including maintenance of male secondary sexual characteristics and development of the prostate gland. Like other nuclear hormone receptors (NRs), AR is a transcription factor that becomes active upon binding to its natural ligand, dihydrotestosterone (DHT).[1] Small molecules that inhibit ligand binding can modulate gene transcription regulated by AR. Environmental exposure to antiandrogens, such as DDT, can cause developmental abnormalities.[2] On the other hand, antiandrogens (flutamide, bicalutamide) currently used to treat prostate cancer present side effects, and drug resistance has been observed with these treatments that therefore provides a compelling need to discover new antiandrogens.

High-throughput screening (HTS) techniques are attractive for both of these needs. Two classes of AR assays have been developed: (1) cell-based transcription assays measuring the inhibition of AR transcriptional activity by small molecules and (2) biochemical competition assays measuring blockade of ligand-binding AR by small molecules. Historically, biochemical assays have been limited by the lack of necessary amounts of pure and functional AR protein whose purification is complicated by low solubility and instability in the absence of androgen.[3,4] Using a His_6-tagged AR-LBD (ligand-binding domain) expressed in *Escherichia coli* in the presence of DHT can overcome these problems.[5]

Although measuring ligand binding by fluorescence polarization (FP) with commercially available fluorescently labeled ligands has become popular, this technique shows limitations in HTS.[6] Both interference with the emission signal from the fluorescent ligand by tested compounds and perturbation of ligand binding and protein function by the fluorescent ligand can be problems. For a robust and broadly applicable biochemical method, radioligands are superior as they more closely mimic the natural ligand. However, radioligands carry with them issues relating to safety and waste disposal. Among radiolabeled ligand-binding assays developed for NRs, only scintillation proximity assays (SPAs) are truly HTS compatible.[7-9] So far, few radiolabeled ligand-binding assays have been described in the 96-well format for AR.[10,11]

Herein we report an AR ligand competition binding assay using SPA 384-well FlashPlates® and liganded AR-LBD protein expressed in *E. coli*. In addition, we show that this assay can be used to measure ligand affinities for other NRs, including the peroxisome proliferation-activated receptor gamma (PPARγ) and the thyroid receptors alpha and beta (TRα and TRβ).

MATERIALS AND METHODS

Materials

Chemicals and materials were purchased from vendors and used without purification: [1,2,4,5,6,7-^{3}H(N)]-5α-Androstan-17

Department of Chemical Biology and Therapeutics, St. Jude Children's Research Hospital, Memphis, Tennessee.

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β-ol-3-one ([^{3}H]-DHT) (110 Ci/mmol) and [^{125}I]-3,3',5-triiodo-L-thyronine [^{125}I]-T3 (779 Ci/mmol) (PerkinElmer, Boston, MA), [^{3}H]-Rosiglitazone (ARC, St. Louis, MO) (50 Ci/mmol), uncoated 96-well polypropylene (3359) and 384-well polystyrene (3573) microplates (Corning Life Sciences, Acton, MA), and 384 Ni-chelate HTS PLUS Flashplates® (PerkinElmer).

Expression and purification of proteins

cAR-LBD (His_6; residues 663-919) was expressed in *E. coli* and purified in the presence of DHT using a modified version of published protocols.[5] Briefly, (pKBU553) was transformed into OneShot BL21 Star (DE3) *E. coli* (Invitrogen) and streaked onto a LB agar carbenicillin (100-μg/mL) plate. A single colony from this plate inoculated a seed culture (overnight, 37 °C). Then, 2 L of 2× LB + 1× carbenicillin and 10 μM DHT were seeded at 0.1 OD and grown at 25 °C with shaking until OD reached 0.6 to 0.8. Expression was induced with 60 μM (final concentration) isopropyl-β-D-thiogalactoside, and cultures were left to grow 14 to 16 h at 17 °C. Cells were pelleted (20 min, 5000 *g*), transferred into a 50-mL conical tube, flash frozen (liquid N_2), and stored at –80 °C. To purify AR, cells were thawed at 4 °C and resuspended in 30 mL of freshly prepared buffer 1 (50 mM Tris [pH 7.5], 150 mM NaCl, 10 μM DHT, 0.1 mM phenylmethylsulfonyl fluoride [PMSF], 10 mg/L lysozyme, Roche Complete EDTA free protease inhibitor cocktail tablet). Cells were lysed by sonication (4 °C, 6 × 2-min cycles with 2-min breaks, 45% amplitude, Branson Digital Sonifier, Branson, Danbury, CT) and clarified by ultracentrifugation (2 × 30 min; 100,000 *g*; 4 °C). TALON resin (1 mL per liter cell culture) was add to a 50-mL conical tube and washed twice with 15 mL freshly prepared buffer 2 (50 mM $NaPO_4$ [pH 8.0], 300 mM NaCl, 10% glycerol, 0.2 mM Tris(2-carboxyethyl)phosphine [TCEP], 0.1 mM PMSF, 2 μM DHT). The protein supernatant was added to TALON resin (40 mL of supernatant for each conical tube) and rotated gently overnight at 4 °C. The resin was pelleted by centrifuging for 20 min followed by washing 5 times with 10 mL buffer 3 (50 mM $NaPO_4$ [pH 8.0], 300 mM NaCl, 10% glycerol, 0.2 mM TCEP, 0.1 mM PMSF, 2 μM DHT, 10 mM imidazole). In addition, resin was washed 5 times with 10 mL buffer 4 (50 mM $NaPO_4$ [pH 8.0], 300 mM NaCl, 10% glycerol, 0.2 mM TCEP, 0.1 mM PMSF, 2 μM DHT, 10 mM imidazole, 2 mM adenosine triphosphate [ATP], 10 mM $MgCl_2$). Elution was carried out in fractions equal to or less then bed volume using buffer 5 (50 mM $NaPO_4$ [pH 8.0], 300 mM NaCl, 10% glycerol, 0.2 mM TCEP, 0.1 mM PMSF, 2 μM DHT, 250 mM imidazole, 100 mM KCl). Protein purity (>90%) was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and analytical size exclusion by fast protein liquid chromatography (FPLC). Protein concentrations were measured by Bradford and bicinchoninic acid (BCA) protein assays. Usually, 6 to 8 mg of protein per liter of cell culture was obtained. The protein was dialyzed overnight against buffer 6 (50 mM HEPES [pH 7.2], 150 mM Li_2SO_4, 10% glycerol, 0.2 mM TCEP, 20 μM DHT) and stored at –80 °C in buffer 6.

Human PPARγ (hPPARγ) was expressed and purified following the procedure above using the following modifications. Cultures were grown up and induced at 22 °C for the same amount of time as above. Induction was obtained with 500 μM of isopropyl-β-D-thiogalactoside. Buffer 1 contained 20 mM Tris (pH 7.5), 100 mM NaCl, 0.5 mM PMSF, 0.5% Triton X-100, and 10 mg/L lysozyme. Buffer 2 contained 20 mM Tris (pH 7.5), 100 mM NaCl, 1 mM imidazole, and 5 mM dithiothreitol (DTT). Buffer 3 contained 20 mM Tris (pH 7.5), 100 mM NaCl, 5 mM DTT, and 1 mM imidazole and was used to wash the beads 7 times instead of 5. Buffer 4 was not necessary in the purification of hPPARγ. Buffer 5 contained 20 mM Tris (pH 7.5), 100 mM NaCl, 5 mM DTT, and 250 mM imidazole. Buffer 6 contained 50 mM Tris (pH 8.0), 25 mM KCl, 2 mM DTT, and 10% glycerol. PPARγ does not require any ligand to remain stable in buffer 6. The average yield was 15 mg per liter of cell culture.

Human TRα and TRβ (hTRα and hTRβ) were prepared using a published procedure.[12]

SPA ligand competition binding assay

All liquid handling was carried out using an automated liquid handling system (Biomek FX, Beckman Coulter, Fullerton, CA). To each well of a 384-well Ni-chelate-coated Flashplate® (PerkinElmer) was added 50 μL of 5 μM NR-LBD in corresponding assay buffer. After a 30- to 60-min incubation, the protein solution was discarded (followed eventually by washes with assay buffer). Then, 25 μL of serial diluted small molecules in assay buffer containing 10% DMSO was added into each well followed by addition of 25 μL of a radioligand solution in assay buffer. The final assay solution contained 5% DMSO. The plates were sealed with clear tape (Millipore® tape multiscreen) and allowed to equilibrate for 1 to 24 h at room temperature or 4 °C. Radiocounts were measured using a TopCount Microplate Scintillation and Luminescence Counter (Packard Instrument Company, Meriden, CT). All data were analyzed using GraphPad Prism 4.03 (GraphPad Software, San Diego, CA); IC_{50} values were obtained by fitting data to the following equation:

(sigmoidal dose response (variable slope)): y = bottom + (top – bottom)/(1 + 10^((LogIC_{50} – x)*Hillslope)),

where x is the logarithm of concentration, and y is the response. Two independent experiments, in triplicates, were carried out for each compound.

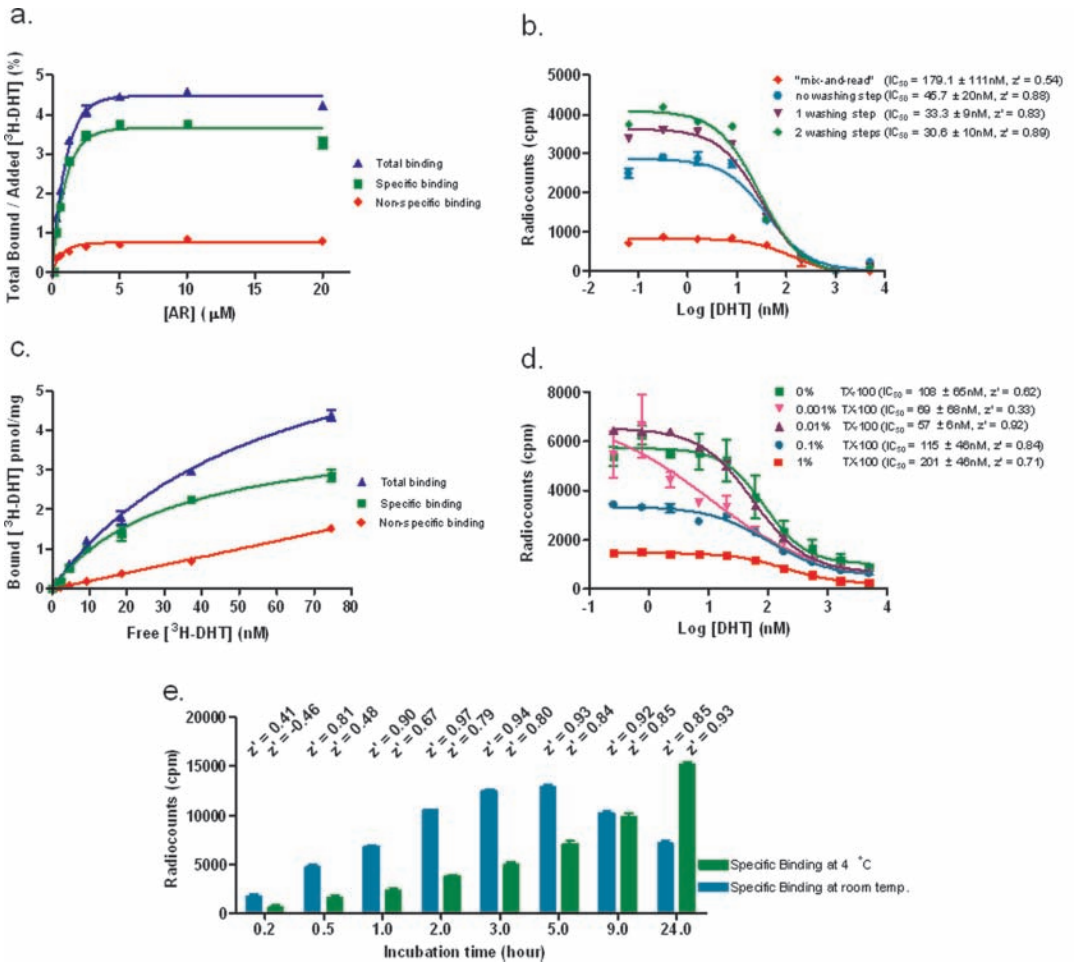

FIG. 1. Optimization of an androgen receptor (AR) scintillation proximity assay (SPA) ligand competition assay. (**a**) Measurements of total binding (AR + 20 nM [^{3}H]-dihydrotestosterone [DHT]), nonspecific binding (NSB: AR + 20 nM [^{3}H]-DHT + 5 µM DHT) and specific binding (SB = total – NSB) for different AR concentrations after 2 washes. (**b**) Effect of washes: SB was measured for experiments carried out with 5 µM AR, serially diluted DHT in the presence of 20 nM [^{3}H]-DHT. (**c**) Saturation binding plot: measurements of total binding (5 µM AR), NSB (5 µM AR + 5 µM DHT), and SB for different [^{3}H]-DHT concentrations. B_{max} = 4.1 pmol/mg, K_d = 31.6 ± 9.3 nM. (**d**) Influence of Triton X-100 (TX-100): SB was measured for experiments carried out with 5 µM AR for incubation step, serially diluted DHT in the presence of 20 nM [^{3}H]-DHT. (**e**) Incubation time course: measurements of SB after different incubation times at room temperature and 4 °C. Corresponding z′ values are specified above columns.

For the AR binding assay, [^{3}H]-DHT was used at a final concentration of 20 nM, and the assay buffer contained 50 mM HEPES, 150 mM Li_2SO_4, 0.2 mM TCEP, 10% glycerol, and 0.01% Triton X-100 (pH 7.2). For the hPPARγ assay, [^{3}H]-rosiglitazone was at 40 nM, and the assay buffer contained 50 mM Tris (pH 8.0), 25 mM KCl, 2 mM DTT, 10% glycerol, and 0.01% Triton X-100 (pH 7.2). For the hTR assays, [^{125}I]-T3 was at 1 nM, and the assay buffer contained 50 mM HEPES, 100 mM NaCl, 1 mM DTT, 0.1% bovine serum albumin (BSA), 10% glycerol, and 0.01% Triton X-100 (pH 7.2).

RESULTS AND DISCUSSION

Optimization of an AR SPA ligand competition assay

A number of assay parameters were optimized. First, we measured total binding (protein and radioligand), nonspecific binding (NSB: protein, radioligand, and excess of unlabeled ligand), and calculated specific binding (total – NSB) for different protein concentrations (**Fig. 1a**). A concentration of 20 nM [^{3}H]-DHT was necessary to minimize background (data not shown). The percentage of bound [^{3}H]-DHT in relationship

Table 1. Summary of IC_{50} Values Measured with a Scintillation Proximity Assay Ligand Competition Assay for Diverse Nuclear Receptors

AR		*PPARγ*			*TRα*	*TRβ*
	IC_{50}, μM		*IC_{50}, μM*		*IC_{50}, μM*	
DHT	0.057 ± 0.006	GW9662	0.23 ± 0.02	T3	0.03 ± 0.02	0.14 ± 0.02
Methyltrienolone (R1881)	0.11 ± 0.02	Rosiglitazone	0.34 ± 0.08	T4	1.4 ± 0.5	4.0 ± 2.0
17β-estradiol	3.2 ± 0.6	Troglitazone	3.70 ± 0.44	GC1	1.6 ± 0.3	0.29 ± 0.11
Cyproterone acetate	2.3 ± 0.4	Lineolic Acid	3.80 ± 0.49	TRIAC	0.18 ± 0.1	0.07 ± 0.05
Bicalutamide	12.0 ± 2.6	Arachidonic acid	4.20 ± 0.53	T4Ac	10.0 ± 2.0	5.0 ± 2.0
Progesterone	5.0 ± 0.7			T3Bz	12.0 ± 2.0	10.0 ± 4.0
Hydroxyflutamide	33.0 ± 8.0					
Flutamide	73.4 ± 29.3					
Dexamethasone	188.5 ± 100.0					

AR, androgen receptor; PPARγ, peroxisome proliferation-activated receptor gamma; TRα, thyroid receptor alpha; TRβ, thyroid receptor beta; DHT, dihydrotestosterone; T3, 3,3′,5-triiodo-L-thyronine sodium salt; T4, L-thyroxine; TRIAC, 3,3′,5-triiodothyroacetic acid; T4Ac, 3,3′,5,5′-tetraiodothyroacetic acid; T3Bz, 4-(4′-Hydroxy-3′-iodophenoxy)-3,5-diiodobenzoic acid.

with the input was low, saturating at 4.5%. On the basis of this result, we used an AR concentration of 5 μM.

Second, we observed that performing the assay directly by mixing the protein along with the unlabeled and radiolabeled ligand ("mix-and-read") led to a high background signal (higher than 50% of total binding), a narrow signal window 1000 cpm (**Fig. 1b**), high standard deviations (IC_{50} = 179.1 ± 111 nM), and a low z′ value (0.54). However, removal of the protein solution prior to the addition of unlabeled and radiolabeled ligand increased the signal substantially. The addition of consecutive wash steps resulted in improved data (IC_{50} = 30.6 ± 10 nM) and assay quality (z′ = 0.89). In addition, we reused the protein solution and carried out the assay the next day without compromising the assay quality (data not shown).

Third, we determined a K_d of 31.6 ± 9.3 nM of this specific ligand receptor interaction by measuring radiocounts for different [^{3}H]-DHT concentrations after incubation with 5 μM AR (**Fig. 1c**). Thus, the [^{3}H]-DHT concentration (20 nM) used was lower than the calculated K_d, although it was 10 times higher than the reported K_d for DHT.[10,11,13] A B_{max} of 4.1 pmoles of bound [^{3}H]-DHT per mg of AR protein was calculated.

Fourth, we focused on the influence of Triton X-100 (TX-100) (**Fig. 1d**) or BSA (data not shown). No effect was observed in the presence or absence of 0.1% BSA. The dose-response curve obtained in the absence of detergent showed high standard deviations among each triplicate and consequently a high variability of the IC_{50} (108.6 ± 65 nM). TX-100 concentrations of 0.01% increased the signal window, as well as gave the best z′ value (0.92) and an IC_{50} value of 56.9 ± 6 nM.

Fifth, we analyzed the time dependency of the signal at room temperature and 4 °C (**Fig. 1e**). Normally, we accumulated data after 5 h, but the assay could be read after 1 h (z′ > 0.5). In both cases, the protein was stable at least for 24 h.

Sixth, we changed the order of addition, with no effect on the results (data not shown); thus, the radioligand can be safely added at the very last step of the assay.

Evaluation of an AR SPA ligand competition assay

To evaluate our AR ligand-binding assay, we investigated several known competitors of DHT and applied this assay procedure to other nuclear receptors: PPARγ, TRα, and TRβ (**Table 1**). The assay conditions were optimized using [^{3}H]-rosiglitazone for PPARγ and [^{125}I]-T3 for TRα and TRβ (data not shown).

For the homologous DHT competition assay, we measured an IC_{50} value of 56.9 ± 6 nM. DHT and R1881 showed the highest affinities followed by miscellaneous steroid hormones. Different classes of PPARγ ligands were investigated. Most active were irreversible antagonist GW9662 and reversible agonist rosiglitazone. The natural unsaturated fatty acids (linoleic and arachidonic acid) exhibited similar activities. Finally, we tested a panel of known T3 competitors. T3 and its analog, TRIAC, showed the highest affinities for TRα and TRβ. The synthetic agonist GC1 and TRIAC exhibited high specificities for TRβ.

Using liganded NRs in these binding assays resulted in generally higher IC_{50} values for competitors compared with the literature values. As calculated K_ds gave us the same range of binding affinities (data not shown), relative binding affinities (RBAs) remain the best choice to draw comparisons with other binding assays. To show the relevance of our radioassay, we plotted log values of measured IC_{50}s against log values of reported binding affinities (**Fig. 2**). We found a statistically significant correlation for the AR (p = 0.0002, n = 6) and TR (p = 0.015, n = 4) receptors, whereas a much less significant correlation was observed for the PPARγ receptor (p = 0.214, n = 3) mainly due to few available comparable published values.[7,13,14]

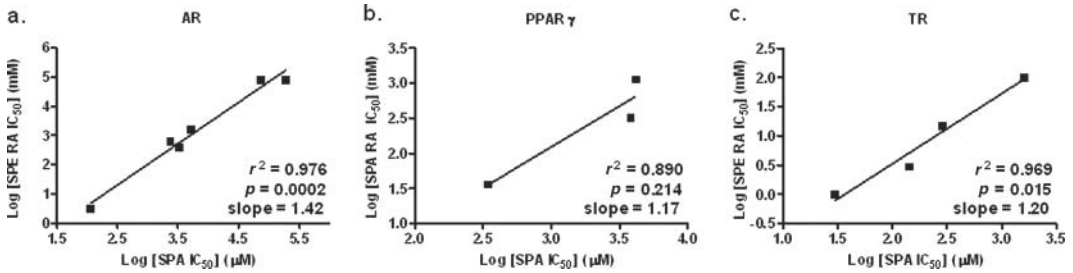

FIG. 2. Correlation plots of IC_{50} values obtained in nuclear hormone receptor (NR) scintillation proximity assay (SPA) ligand competition assay using FlashPlate® versus literature values (Solid phase extraction radioassay [SPE RA]). (**a**) Androgen receptor (AR), (**b**) peroxisome proliferation-activated receptor gamma (PPARγ), and (**c**) thyroid receptor (TR) SPA ligand competition assays using FlashPlate® versus reported radioassays.[7,13,14]

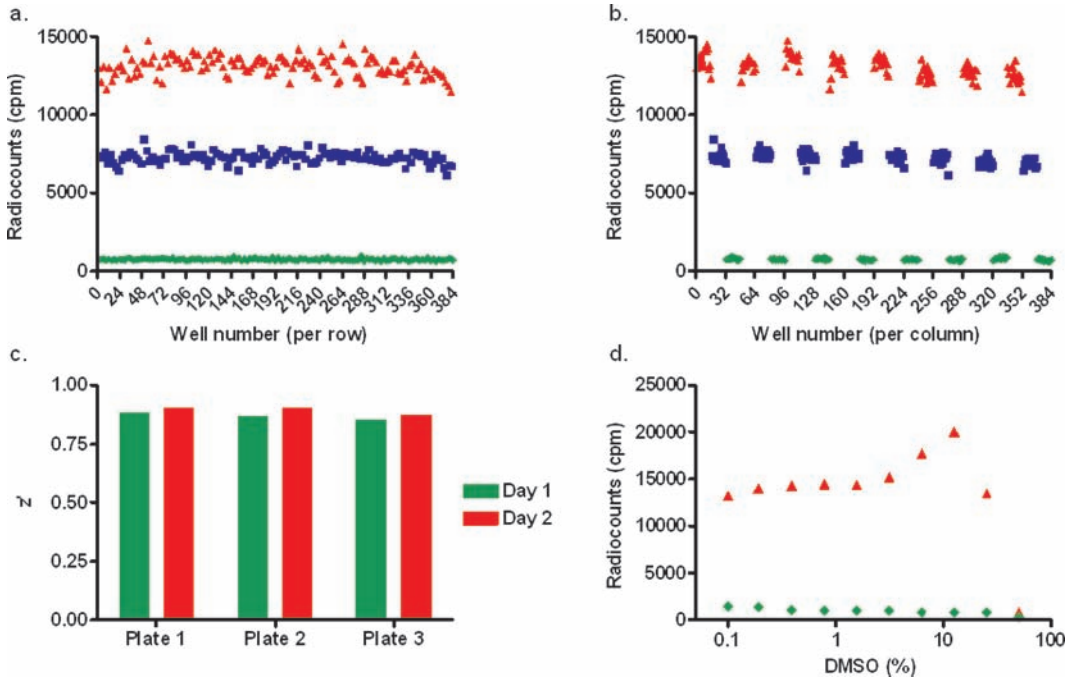

FIG. 3. High-throughput screening validation. Plate geographic effects investigation across (**a**) rows and (**b**) columns. Dihydrotestosterone (DHT) was assayed at different concentrations in the presence of 20 nM [^{3}H]-DHT (▲ DMSO, ■ [DHT] = 50 nM, ◆ [DHT] = 5 μM). (**c**) Assay reproducibility: comparison of z′ values determined by using 2 independent assays performed in triplicates. (**d**) Assay stability: influence of DMSO content on radiocounts.

Validation of an AR SPA ligand competition assay for HTS

To investigate if the optimized AR SPA ligand competition assay can be automated for HTS, we followed validation protocols from the National Institutes of Health (NIH) Chemical Genomics Center (NCGC).[15] First, we carried out a plate uniformity assessment. Therefore, after preincubation with 5 μM AR solution, 3 different FlashPlates® were treated with DMSO (high signal), 50 nM DHT (medium signal), and 5 μM DHT (low signal) in the presence of 20 nM [^{3}H]-DHT following the published plate layouts. Radiosignal measurements were read after 5 h (**Fig. 3a,b**). Plotting the radiocounts against the well number by row, we observed a linear relationship between radiocounts and well number. This behavior excluded the presence of drift or edge effects. In addition, we plotted radiocounts against the well number by column. The clustering of values indicated no major variation of the measured signal depending on the geographic position. The experiment was repeated twice, and z′ values were found between 0.85 and 0.92 for all plates, confirming the integrity of independent assays (**Fig. 3c**). Finally, we noticed that DMSO concentrations were tolerated up to 5% without a change in signal (**Fig. 3d**).

In summary, we have described a ligand competition assay for the androgen receptor using 384-well FlashPlates® and purified liganded AR-LBD. The "mix-and-read" process led to very low accuracy and z′ values. We recommend the removal of unbound AR-LBD prior to the addition of small molecule and [^{3}H]-DHT. This process allows protein recycling. In addition, we were able to confirm the robustness of signal from 1 to 24 h, allowing the detection of slow binders. Drift experiments showed excellent homogeneity and reproducibility. All z′ values measured in the optimized conditions are higher than 0.85, whereas fluorescence polarization methods tend to perform around 0.6. Although absolute measured IC_{50}s of known binders vary from reported values, we observed a strong correlation between IC_{50}s determined by both methods, indicating that this method provides reliable measurement of relative binding affinities. The addition of the radioligand as the last protocol step, followed by sealing with clear tape, decreases significantly the risk of contamination. Finally, the cost per data point was relatively low in comparison with other NR binding assays due to the 384-well format and in-house production and recycling of proteins. Overall, we are convinced that this assay can be fully automated and used for HTS purposes.

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Address correspondence to:
R. Kiplin Guy
Department of Chemical Biology and Therapeutics
St. Jude Children's Research Hospital
262 Danny Thomas Place
Memphis, TN 38105

E-mail: kip.guy@stjude.org